THE INFLUENCE OF AGE AND PROLONGATION OF LARVAL LIFE ON THE OCCURRENCE OF SPONTANEOUS MUTATIONS IN DROSOPHILA.

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Thesis for Ph.D., University of Edinburgh.

1940.

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I.

THE INFLUENCE OF AGE ON THE OCCURRENCE OF SPONTANEOUS MUTATIONS IN DROSOPHILA.

I. Introduction

A living species is constantly under pressure of the mutation process tending to produce changes in the characteristics of the organisms. The magnitude of the mutation pressure is evidently a problem of prime importance for any theory of evolution. The works of present day geneticists may throw a new light on the observations made long ago by systematists and palaeontologists, showing that the pace of evolution is not alike in all organisms. Some groups seem to possess an unlimited store of variation and evolve rapidly, while others are conservative and undergo no change during geological epochs. A classical example of evolutionary conservatism is the brachiopod genus Lingula, which was already living in the seas of the Palaeozoic period and yet has neither changed nor become extinct in our time. While factors affecting the rate and type of selection that occurs are no doubt of great importance in the origination of such differences in some of these cases, an adequate analysis requires that possible variations in the mutation pressure/

pressure be disentangled from the selective factors, so that the influence of variations in the mutational factor alone may be assessed.

Mutations have been observed under controlled conditions in many animal and plant species. Sometimes they arise in strains not known to have been exposed to treatment by mutation-producing agents; such mutations are designated "spontaneous mutations". Mutants are, on the whole, rare; moreover, they arise mostly as single individuals among masses of unchanged representatives of a strain. Mutations arise at all stages of the developmental cycle - in gametogenesis before and after the reduction division, in gametes and in somatic tissues of varied kinds.

The frequency of spontaneous mutation varies greatly with the character in question, with different organisms and, if individual genes are considered, with the gene itself. In Drosophila melanogaster, Muller (1928) found a rate of nearly 1 in 100 for the appearance of lethal genes at a particular locus in the second chromosome, while in the same animal Patterson and Muller (1930) estimated the rate of mutation at the white locus as only about 1 in 400,000 to white/

white and 1 in 600,000 to other allelomorphs. In Zea, Stadler (1932) found rates of mutation from 1 per 1,000 to less than 1 per 1,000,000 pollen grains for seven colour genes. In human beings Haldane (1935) estimated the rate of mutation for the gene for haemophilia as 1 in 50,000 life cycles.

These results indicate that the frequency of mutation of genes varies greatly not only with the animal or the plant in question but also with the constitution of the gene itself. In experiments reported by Muller (1928), variations in the frequency of sex-linked lethal mutations ranging from more than 1 in 100 to ca. 1 in 1,000 were found in different experiments, utilizing different stocks. Demerec's findings (1937) with 15 wild type stocks of Drosophila melanogaster show similar significant variations of the rate of lethal mutation from one stock to the other. Amongst all these 15 types, a Florida stock had the highest rate of mutation (ca. 1 in 100). He infers from mutation tests on the descendants of crosses of this stock to stock containing "markers" that the Florida stock carried in the second chromosome a recessive gene which/

which was responsible for the high rate of mutation .

Such variations in the "spontaneous" mutation frequency serve to emphasise the need for obtaining more information concerning the factors underlying the process of mutation. After all, the name "spontaneous", when applied to any natural process, obviously constitutes only a thinly-veiled admission of ignorance of the real causes of the phenomenon in question. This applies fully to the causes of spontaneous mutation. For years therefore the attention of workers in the field of mutation has been centred on attempts to find agents modifying or varying their frequency.

The first and most important attempt in this direction was the discovery by Muller (1927) which disclosed X-rays as the first and foremost agent known for inducing mutation. Since this discovery a considerable amount of work has been done to see how far gene mutation in general depends on radiation. The findings of Hanson, Heys, and Stanton (1929, 1931, 1932), Muller (1932-1938), Oliver (1930 , 1932, 1934), Stadler (1930-1939), Patterson (1931), Serebrovsky and Dubinin (1930)/

(1930), Stubbe (1937), Sax (1938), and Timofeeff-Ressovsky (1931-1937) have demonstrated that (1) the frequency of the induced gene mutations is directly proportional to the total energy absorbed from the high energy radiation, (2) there is little or no temperature coefficient for X-ray induced mutations, (3) differential susceptibility is found in different types of cells or stages of development, (4) there is no differential effect of the various wave lengths in the X-ray-gamma-ray range and (5) X-rays cause translocations, inversions and deletions of chromosome segments as well as "gene mutations". On the basis of such findings it was easiest to think that the impacts of released electrons must be the primary causative agents in producing mutations, an individual successful impact resulting in an individual gene mutation.

X-ray produced mutations may be classified into gene mutations and inheritable gross and minute rearrangements of chromosome parts containing many genes, all in comparable frequencies at the doses commonly used, in contrast to the "spontaneously" produced mutations, the vast majority of which are gene mutations. Even at/

at very low doses of radiation, where the frequency of gross rearrangements is disproportionately low (owing to their frequency varying exponentially with the dose), there are still a comparable number of minute rearrangements and of "gene mutations" not involving a detectable rearrangement. But the gene mutations occurring "spontaneously", i.e. without radiation artificially applied, are not accompanied by any such relatively large number of minute rearrangements. Now if gene mutations and rearrangements were not two different types of mutations, it is quite improbable that there should be this difference in their relative frequencies in the presence and absence of radiation.

It has been inferred by Muller (1932, 1937, 1940) and by Timofeeff-Ressovsky, Zimmer and Delbrück (1935), that an ionization, in order to produce a mutational effect, involves an intermediary course of events, dependent somehow upon the peculiarities of the biological and chemical processes of the cell. These intermediate processes between the initial ionization and the mutation itself might conceivably be of various kinds. This is in harmony with the idea that/

that influences other than radiation may also be able to induce gene-changes by affecting the chemical and biological processes of the cell.

The elaborate calculations of Muller and Mott-Smith (1930) showed clearly that the "spontaneous" mutations were the effects of something other than natural high-energy radiation. This fact was supported by the work of Timofeeff-Ressovsky (1931) and of Efroimson (1931). Muller and Mott-Smith calculated that the radiation received by the flies in the entire period of their reproductive generation (from the germ cells of the parent to the germ cell of the offspring) is at least 1,300 times too low to explain their "spontaneous" mutation rate on the principle of a linear relation. Some biologists had thought, however, that radiation might be more effective at low intensities. The recent work of Ray-chaudhuri (1939) proved, however, that the effectiveness of radiation even at the low intensity of .01 r/min. is still only proportional to the total dosage (ionization) received. This would leave little ground for the theory that natural radiation produces an appreciable proportion of the natural mutations in Drosophila.

The/

The induction of mutation by thermal and chemical influences further supports the fact that radiation is not the usual causative agent for spontaneous mutations in organisms in nature. Early findings of Muller and Altenburg (1919), and of Muller (1928), confirmed and extended by Timofeeff-Ressovsky (1935), showed that a moderately raised temperature, applied over a considerable period, induces an increase in the mutation frequency. This increase was interpreted as representing an application, to mutation, of Van t' Hoff's rule that a rise of 10°C causes an increase usually of 2 to 3 times the speed of chemical reactions.

In this connection, Delbrück (1935), collaborating with Timofeeff-Ressovsky, points out that, as has been well known to physical chemists, the amount of increase in the rate of reaction, caused by a rise of temperature, depends upon the rate of the reaction itself, at any one given temperature. Now when, in the results of Timofeeff-Ressovsky (1935) and of Muller and Altenburg (1919, 1928), the time factor and probable error are properly taken into account, it is found that they fall well within the/

the range of the exceptionally high value of Q_{10} (5 to 6 times) which is to be expected for chemical reactions of such very low speed as the mutation frequencies represent. In other words, the results fit in with the conception of individual mutations as being due to individual microchemical accidents, like the changes of individual molecules in a reacting mixture, the total mass of which obeys the laws of mass changes.

While tracing the influence of temperature upon the mutation, it may be noted that the curve for the rate of mutation appears to rise more sharply at higher temperatures. The works of Muller (1928), Rokitzky (1930), Plough (1932, 1939), Timofeeff-Ressovsky (1935) and Zuitin (1938) showed that the frequency of mutation is raised considerably by abnormally high temperature ("shocks") applied over a short period, (although the results of Ferry, Shapiro and Sidoroff (1930) and of some others not employing the exact quantitative technique of the lethal mutation rate, are negative as regards such a temperature effect). It may be remarked in this connection, however, that the very extreme increases in the visible mutation rate reported by Goldschmidt/

Goldschmidt (1929) and by Jollos (1930) have not been confirmed and Goldschmidt (1939) at least no longer regards them as effects of temperature. This disproportionate effect of very high and also of very low (Kerkis, 1939, Zuitin, 1939) temperatures indicates a complicated set of reactions in which more than one process take part. It is to be expected that, when partially lethal temperatures in either direction have been reached, abnormal chemical processes of various kinds will set in within the cells, and that, if the reaction of mutation is a chemical one, some of these abnormal chemical processes may tend to affect the mutation reaction.

On such a chemical conception of the mutation process it is also not surprising to find an influence of certain chemicals on the frequency of mutation. The works of Sacharov (1936), Lobashov (1935) and Magrzhikovskaja (1938) do seem to give evidence of a moderate influence of certain chemical treatments upon the mutation frequency in Drosophila, but without exact information concerning the nature of the controls, etc., and until the results have been confirmed in additional series of data, they do not appear very securely/

securely founded.

Navashin (1933) has shown that changes occurring towards the end of a period of ageing in seeds lead to an increased frequency of mutation. These mutations are however largely structural changes in chromosomes, and until it can be shown that gene mutations form an appreciable proportion of them and follow the same rule, these results can hardly be regarded as pertinent to our present problem.

Problem.

As the previous discussion indicates, the factors which cause spontaneous mutations may for convenience be classed as (a) external agents and (b) the physiological state (including chemical conditions) of the cell as influenced by such circumstances as degree of activity, amount of cellular proliferation, etc. As a first step in the study of how far variation in the latter processes influences the process of "spontaneous" gene mutation, it was considered desirable to investigate the effect of age, sex, and degree of anabolic activity involving gene reproduction on mutation, while keeping the external factors as constant/

constant as possible. While the work on this problem was under way, papers appeared by Shapiro (1938) and by Sacharov (1939) reporting work of a similar type. These will be discussed later, in connection with the discussion of our own work, and of some work of Timofeeff-Ressovsky (1937) on the effect of ageing spermatozoa (which also came to our attention after our own work was under way).

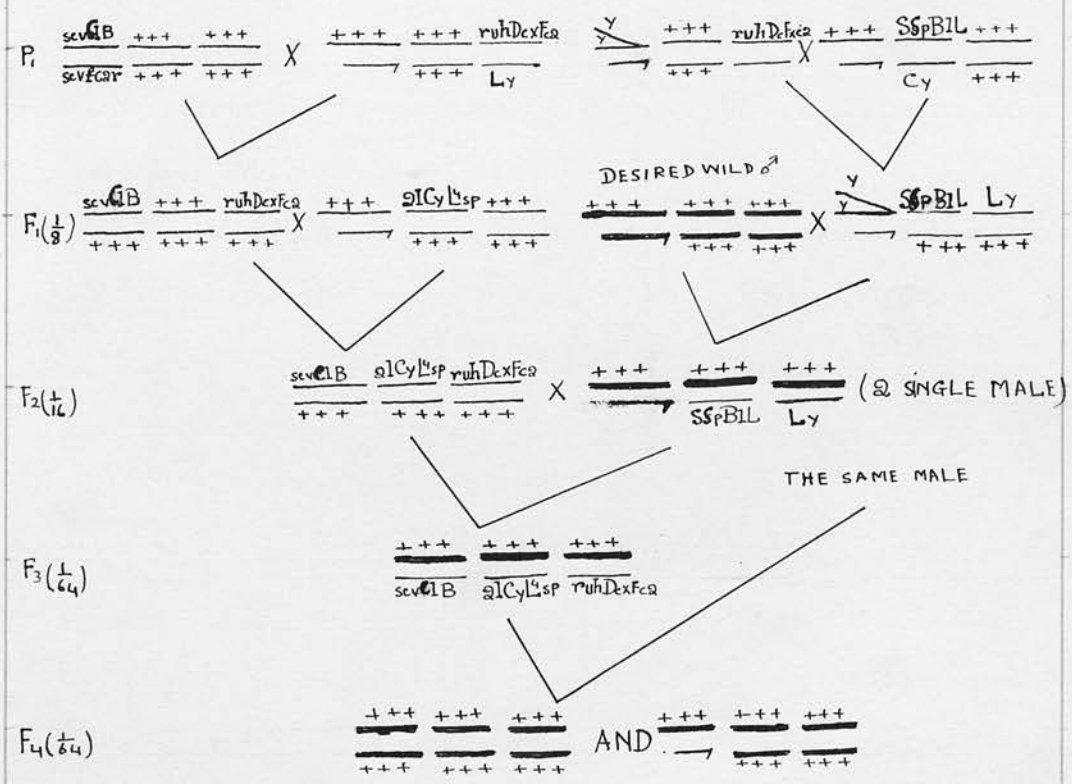
II. Methods and Material.

(a) The preparation of homozygous wild type stocks and testing their rate of lethal mutations.

Earlier work of this and other laboratories had shown that the mutation rate, even for the same general stock of flies, may vary from time to time. In experiments like the one projected by us, where the rate of spontaneous mutation is to be studied, such large numbers of cultures are required because of the low frequency of such mutations that it is desirable to select a stock which naturally gives a fairly high mutation frequency, and perhaps an even more important qualification of such a stock is the giving of as constant a rate of mutation as possible when the culture conditions are sensibly uniform. The latter end might be gained by the construction of a genetically homozygous stock.

These considerations urged strongly the necessity of getting a homozygous stock having high mutability. Three wild-type stocks, Florida, Amherst-3, and Amherst-10 - the latter two obtained from Professor Plough - which in previous work of Demerec, Plough and others had in some experiments shown a rather high mutability, were chosen/

chosen. These were made as homozygous as possible by the following scheme and were thereupon tested for their frequency of sex-linked lethal gene mutations.



Note:- The chromosomes with the dominant markers give very little crossing over and allow the selection of the desired combinations. The symbols are the standard ones for well known genes, keys to them being given in D.I.S. 9 and elsewhere.

Ten/

Ten crosses of each of these stocks were made in the "F_1" generation of this system of crosses. Out of these, 2 of the Florida, namely the 4th and 5th, 2 of the Amherst-3, namely the 4th and 6th, and 1 of the Amherst-10, namely the 9th, were successfully carried through to the fourth generation in the manner shown. The reason for the failure of the majority of the lines was that in the F_2 generation the expected proportion of flies of the required type was only 1/16 of the total zygotes produced, in the F_3 only 1/64, and in the F_4 again 1/64. In Amherst-3 one more line was at first obtained, but it died before being bred. Each line of these stocks was then bred and the frequency of sex-linked lethals was tested out for each.

For these tests the ClB method for detecting sex-linked lethals was used. The males (P_1), not over one day old, were taken and allowed to inseminate $\frac{\text{sc v ClB}}{\text{sc v f car}}$ virgin females for 5 days. These vials were called "P_1", following our custom of designating the generation of a vial by that of those flies which are put in it as parents, not of those which hatch from it. The P_1 males in them were discarded and the

P_1/

P_1 females transferred once more to other vials for 5 days for the purpose of obtaining a second "brood". Thereafter the P_1 females also were discarded.

The F_1 females of constitution $\frac{sc\ v\ ClB}{+\ +\ +}$ were placed individually in vials and crossed with males of the constitution $\underline{sc^8\ B\ w^a}$ (these vials were called F_1). Amongst the progeny in such vials, half the males die because of the lethal gene in the ClB chromosome; this produces a sex ratio of 2 females to 1 male. If then a sex-linked lethal has arisen in the other chromosome ($\underline{+\ +\ +}$), all the males die. Occasionally one or two non-disjunctional males appeared in the vials to be scored as lethals but these were recognized because they received their X-chromosome from their father which had been distinguished by markers (usually $sc^8\ B\ w^a$, but in cases where the mothers were not virgin sc v f car might also appear). Confirmation of such lethals was carried to the following generation by taking the F_2 females of $\frac{+\ +\ +}{sc^8\ B\ w^a}$ constitution (which are recognized by being non-scute (sc) and having eyes of a less extreme Bar (B) type than the females of $\frac{sc\ v\ ClB}{sc^8\ B\ w^a}$ constitution) and crossing them/

them with $\underline{sc^{Sl}}$ males.

The following are the results of these test experiments:

	Stock	Chromosomes examined	Lethals	%
1	Florida (5)	1298	13	1.00
2	Florida (4)	1664	17 (4 together from the same P_1 male)	1.02
3	Amherst-3 (4)	1009	8	.79
4	Amherst-3 (6)	1460	9	.62
5	Amherst-10 (9)	2042	8	.39

It will be seen that, except for Amherst-10 (9), the differences are not significant and all the stocks gave a rather high mutation frequency. F1(4) gave the highest sex-linked lethal frequency of all but as 4 lethals came from the same original P_1 male the error of the resulting value was greater. F1(5) was therefore decided upon for use in the main experiment. It was soon found, however, that it had a much lower viability and fertility than the other stocks. Therefore F1(4) was finally chosen as likely to be best adapted for our purposes.

(b)/

(b) Other stocks used.

The following are the stocks used in the present experiment:

1. Florida 4: wild type, relatively homozygous.
2. sc^8 B w^a males maintained by crossing them with females homozygous for yellow ($\frac{y}{y}$) having attached-Xs.

   The sc^8 B w^a X-chromosome contains the mutant genes $scute^8$, Bar eye and the apricot allele of white eye.

   sc^8 - The sc^8 mutation gives a very slight reduction of bristles, very irregularly distributed, and often accompanied by an increase in the number of some bristles and hairs, especially the sternopleurals and the bristles on the longitudinal veins ("Hw effects"); its expression considerably overlaps the wild type. Inseparable from $scute^8$ is a very long inversion having its breaks between ac and sc very near the left end and between bb and sp-a, very near the right end of the chromosome. With an X-chromosome of normal structure it gives only double crossovers.

   The dominant Bar eye, B (locus 57), and the recessive apricot eye, w^a (locus 1.5), are too well/

well known to require description here.

3. sc^8 sn w^a males were maintained by crossing with females of the type $\frac{y}{Y}$. They have the mutant genes sc^8, w^a and singed (sn).
   The recessive singed bristles, sn (locus 21.0), cannot exist in homozygous stock, as the homozygous females are entirely sterile.

4. sc d149 v f: Stock homozygous for the three recessive mutant genes indicated and for the inversion (d149), all in the X-chromosome.
   sc: sc^1 (locus 0.0±): most scutellar and some other bristles absent, in definite pattern.
   d149: Inversion, much used in balanced stocks of male-sterile or male-lethal mutants, gives practically no crossovers between yellow (0.0) and garnet (43.0).
   v: vermilion eye (locus 33.0).
   f: forked bristles (locus 56.7).

5. $\underline{sc^{S1}}$, sc^{S1}: This is an extreme sc allele, practically sterile and of low viability in the homozygous female. It is associated with a long inversion similar to that of sc^8, but having the left break just to the right of sc, the right break being between bb and sp-a. Within the long inversion is a smaller one, somewhat smaller/

smaller than that of dl49. The chromosome is a useful balancer for the whole X.

(c) Experimental technique.

(1) Division into groups.

There were three groups in this experiment:

1. "Control"
2. "High anabolism"
3. "Low anabolism"

1. For the "control" only the first eggs laid by newly hatched (0-5 day old) wild type "P_1" females, after being inseminated by sc^8 sn w^a males, were used. The females developed from these eggs (laid in the first 3 days after mating) were tested to find the frequency of sex-linked lethal mutations contained in them.
2. "High anabolism" - the P_1 wild type females and sc^8 sn w^a males transferred from the control vials were aged together, on the rich diet of fresh standard yeasted food (formula of Offermann and Schmidt, 1935 D.I.S. 3), to give them optimal nourishment, the chance to mate as often as they naturally wish, and to lay as many/

many eggs as possible. They were transferred to fresh standard food approximately every four days for 26 days and only the females developing from eggs laid after the 26th day (and to about the 30th day) were used for the lethal tests of this group.

3. "Low anabolism" - "Control" P_1 females, after being inseminated and bred for 3 days on standard food as before, were separated from the P_1 males and kept on a barely maintenance diet of syrup food (Offermann and Schmidt, 1935 D.I.S. 3) for 25 days, after which they were again put on standard yeasted food for the laying of the eggs from which the F_1 females developed which were to be used for the lethal tests of this group.

(2) Method of detecting sex-linked lethal mutations.

In all these three groups sex-linked lethals present in the F_1 females could be recognized by virtue of the markers differentiating the paternal X-chromosome (sc^8 sn w^a) from the maternal one (F14 wild type). Since, however, the maternal chromosome might already have contained a recessive lethal gene, it was necessary to exclude/

exclude this possibility by finding the sex-ratio produced in the previous generation, using flies only from the cultures in which it was clear that no lethal ratio (2 females:1 male) was present. Therefore, instead of starting the individual cultures with matings of the wild type "P_1" females with sc^8 sn w^a males, it was necessary to make up individual cultures of the parents of the "P_1" F1 4 females, these parents being designated as "P_0"; at the same time individual sc^8 sn w^a males and  females were maintained in vials. In this P_0 generation, then, single male and single female flies of F1 4 were mated in individual vials (called "P_0 vials"). In the case of sc^8 sn w^a, one male and two females with attached-X's were kept in each vial. In the case of F1 4, a count was made of the numbers of males and females to see that the females' chromosomes had not carried a lethal in the previous generation. Unless the number of females was significantly and decidedly less than double that of the males, the mother in such a vial was considered to be under suspicion of containing a lethal gene and all the flies in such a vial were discarded.

The/

The F1 4 females (P_1) from the "P_0 vials" which gave evidence of the mother (P_0) having had no lethal gene, were collected while virgin and mated with sc^8 sn w^a males collected at nearly the same time. Among the progeny (F_1) in these vials (denoted as "P_1 vials"), all the females were of the constitution $\frac{+\ +\ +}{sc^8\ sn\ w^a}$. It was not necessary to collect these females as virgins. In order to reject the cases in which a sex-linked lethal might have arisen between the "P_0" and "P_1" generations, the sex-ratio was noted once more among the progeny (F_1) in the "P_1" vials.

To determine the presence of a sex-linked lethal in F_1 females (of composition $\frac{+\ +\ +}{sc^8\ sn\ w^a}$) they were mated with the sc^8 B w^a males. Amongst the progeny of such F_1 flies, two main types of males were expected: wild type and sc^8 sn w^a. In case a lethal mutation were present on either of these chromosomes, however, the corresponding kind of males did not appear. If only one or two males of any one kind appeared, the case was recorded as a probable semi-lethal, and it, like the suspected lethals, was tested further, as shown below.

All/

All the apparent lethals were subjected to further tests for confirmation. Lethals on the wild type chromosome were to be found in the $\frac{+\ +\ +}{sc^8\ B\ w^a}$ females of the F_2 generation. If in the F_3 generation there was no wild type male, the lethal was confirmed. For confirmation of the lethals on the sc^8 sn w^a chromosome, the F_2 female with the constitution $\frac{sc^8\ sn\ w^a}{sc^8\ B\ w^a}$ was crossed with $\frac{sc^{sl}\ w^a\ sc^4}{}$ males and the lethal was confirmed by the effect on the sex-ratio and on the relative numbers of sons (F_3) of the different classes.

Sometimes, although rather rarely, one or two non-disjunctional males appeared in the vials to be scored as lethals for the wild type chromosome. If they were derived from a wild type father, the case in question could not be distinguished from a semi-lethal except by the above-mentioned confirmation test in the next generation.

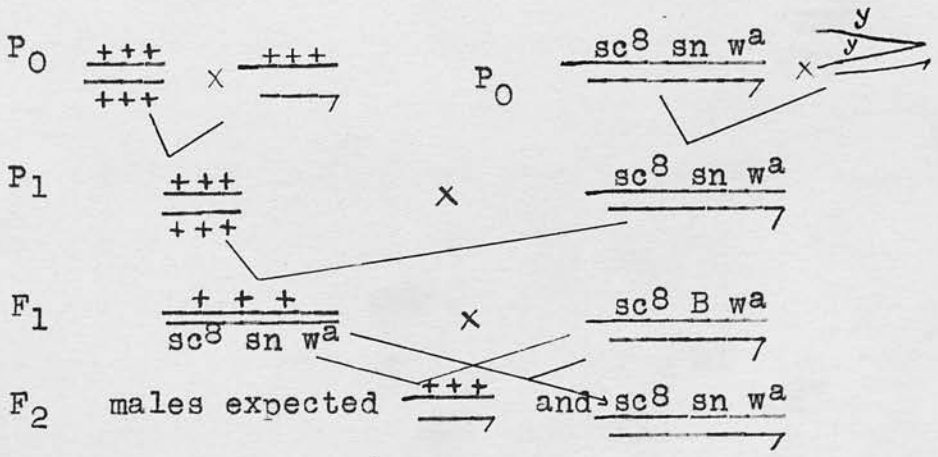
(3) Location of the lethals.

The P_1 males and females were given individual numbers running serially. If more than one lethal in F_1 arose from the same source (P_1 number) their loci were determined in order to ascertain whether or not they were independently arisen lethals/

lethals. To find out the locus of the lethal in the wild type chromosome, the F_2 females of constitution $\frac{+\ +\ +}{sc^8\ B\ w^a}$ were crossed to $\frac{sc\ v\ f\ car}{}$ males and the resulting F_3 females ($\frac{+\ +\ +}{sc\ v\ f\ car}$) were crossed with any except wild type males. By the crossing-over values shown among the F_4 males the lethal was then located.

In the case of lethals found in the $sc^8\ sn\ w^a$ chromosome, the females of type $\frac{sc^8\ sn\ w^a}{sc^8\ B\ w^a}$ were crossed by any male and the crossing-over values shown among the F_3 males were used for locating the lethals.

The scheme of crossing is shown below.



Complete absence of either kind of F_2 males indicates lethal.

(4)/

(4) Culturing methods.

In breeding the "P_0" flies, equal numbers of the cultures termed "P_0 vials" were taken for getting the F1 4 types of P_1 flies and the sc^8 sn w^a type. In the case of the F1 4 cultures of P_0, one male and one female were mated together and every P_0 vial was numbered and dated. In the second group of P_0 vials, each had one sc^8 sn w^a male and two $\frac{y}{y}$ females. The desirability of two females is due to the fact that only half the fertilized eggs of such females result in viable flies. In both cases flies not more than three days old were taken. These P_0 vials were kept in the constant temperature room (23°C). After 4 days, all these P_0 flies were transferred to new P_0 vials. Extra males or females were added in case any of the original sc^8 sn w^a males or $\frac{y}{y}$ females had died. In case of death of the male or female of the F1 4 P_0 vials, the remaining parent was discarded and a new P_0 vial was started in its place, with a new number. These reculturings were repeated every four days.

When the "P_1" flies in the above vials were about to hatch, the vials were carefully watched for the emergence of the first flies, and virgins/

virgins were collected from the F1 4 vials. Collection of such virgins was carried on for two or three days from each F1 4 vial, and the number of males and females hatching was recorded at the same time. Virgins were not collected after three days, but the counts of males and females hatching were continued for the determination of the sex-ratio, in order to ascertain that there was no lethal already present. The flies from all P_0 vials giving any doubtful or suspicious sex-ratio were discarded.

The virgin "P_1" females from any given F1 4 P_0 vial were placed singly in new vial cultures (P_1) with fresh yeasted food, dated, and numbered with the same "P_0" number as that given to their parent P_0 vial, plus an additional number, characterising them in their own generation. Vials containing sister P_1 females derived from the same P_0 vial were bound together in one group. One P_1 male, of the same age as the female, taken from the P_0 vials of the cross $\underline{sc^8\ sn\ w^a} \times \overset{Y}{\underset{Y}{\rightrightarrows}}$ that had been set going at the same time as the P_0 vial that gave the female, was put into each P_1 vial containing one F1 4 P_1 female. The males used were always collected/

collected during the same range of time as the females, so they were of almost the same age as the females.

P_1 males and females were kept together for 72 hours to be sure that almost all the females were inseminated. P_1 vials of each P_0 group were then divided into two lots: in one lot each pair (female and male) was kept together on standard yeasted food (for "High anabolism", i.e. high rate of multiplication of their germ cells) and in the other lot the females were separated from the males and put on syrup food ("Low anabolism"). The P_1 vials into which the flies of both lots had been put for the first 72 hours were used as the control group.

The P_1 flies in the case of high anabolism were transferred every 4th day to freshly prepared yeasted food, and this procedure was continued up to 26 days after their first being put in the P_1 vial for mating. The last period of change was only of 3 days and the first from the control P_1 vials was also of 3 days. The vial of standard yeasted food into which they were transferred on the 26th day was not discarded, but the eggs laid in it were allowed to develop into/

into the F_1 flies which furnished the material on which the lethal tests of the high anabolism group were conducted.¶ In the case of the low anabolism lots, the females were transferred to fresh syrup food every 4 days up to 25 days when they were finally placed (still without males) on standard yeasted food again for the laying of the eggs from which the F_1 of that group were to be derived. In this case also the first period of change from the control P_1 vials was of 3 days and the last of 2 days only.¶ In the case of both the high and the low anabolism, the F_1 flies in question began to appear about 35 days after the mothers had first been inseminated, whereas in the case of the control they began to appear 10 days after the first insemination. In the high anabolism group, the ageing of the flies took place under the same conditions of feeding, etc., as obtained during the breeding of the control group. Moreover, the presence of the male with the female may have provided a stimulus for continued egg laying and egg production, since it is known that females which have never mated, at least, are considerably inhibited in egg laying. At the same time, the ejaculation of sperm allowed the sperm that had matured/

matured earlier to become used up for the earlier laid eggs, so that the F_1 finally tested were derived from male germ cells that had undergone considerable multiplication (as spermatogonia) orfrom those which were dormant (without multiplication) during the period in which the transfers of P_1 were being made. The difference between the high anabolism group and the control may thus, in a sense, be considered as merely one of age.

In the low anabolism group, sperms received during the first three days were retained inside the spermathecae of the female while the P_1 female was being kept on syrup food. Thus there was no chance for multiplication of the male germ cells in this group during the process of ageing. The syrup food was too poor in protein content to allow the production of any but a very few, mostly abortive eggs. Thus cell growth and multiplication in the ovary also were in all probability almost completely checked and the ageing of germ cells of both sexes in this group formed a marked contrast to that in the high anabolism group in respect of the activity of cell growth and division.

In order to keep the factor of

temperature/

temperature nearly constant, the flies of all the groups from the P_0 to the F_1 generation were always kept in a thermostatically controlled room, at $23^{\circ} \pm 1^{\circ}$ C.

In the high anabolism group difficulty was caused by the flies tending to become entangled in the food and dying. Males at the onset of age were more susceptible to this difficulty than the females. In the low anabolism group also the syrup food sometimes became sticky and the flies began to die. If this condition was not detected soon after its onset, it caused the loss of a considerable number of flies. The number of eggs laid was, as expected, low in both the aged groups, but lower in the case of the low anabolism group. In the beginning the P_1 flies were divided into two equal lots for the high and the low anabolism treatments respectively, but when it was noted that the low anabolism flies produced a lesser number of eggs, the P_1 flies were divided in the ratio of 2 for the high anabolism to 3 for the low anabolism.

As previously mentioned, a count of the numbers of males and females was again made in the progeny of the P_1 vials, to guard against the use of/

of flies derived from P_1 in which a lethal had already been present, and in all suspected cases the flies were rejected. The F_1 females hatching from the remainder of the P_1 vials, mated individually with sc^8 B w^a males, were again grouped into batches of sister vials, corresponding this time to the P_1 vials from which they had been taken. Each batch was given the number of both the P_0 and the P_1 vial from which it had been derived. When the F_2 hatched from these "F_1 vials", wild type males and sc^8 sn w^a males were looked for. The absence of either kind was recorded as indicating the existence of a lethal, carried by the corresponding chromosome, and crosses of the F_2 females were in such cases made in the manner previously mentioned, to confirm the existence of the lethal.

The recording of the P_1 vial from which the F_1 had been derived was of help in indicating which lethals might have had a common origin. When more than one sex-linked lethal was found among cultures derived from the same P_1 vial, it was evident that they might have represented the same original lethal. This supposition was confirmed if their locus was found to be the same. For/

For some purposes, it is desirable to know the total number of cases of origination of mutations (together with complementary data on the number of individuals in which each mutation was found) rather than the total number of mutant individuals found, and so both values are given in our tables. Only the latter value can be regarded as an index of the mutation rate, but the size of its statistical error, strictly speaking, should be computed by taking the former value into account.

In the case of the control group care was taken to obtain as constant a number of F_1 females from individual P_1 vials as possible. Sometimes the occurrence of bad cultures did not allow us to maintain this constancy. In the case of the high anabolism and low anabolism groups it was difficult to keep such numbers constant because of the very few F_1 females being found from P_1 vials. Whatever number of F_1 females was obtained was used for these groups. In the case of the control group, there were many P_1 vials, all of which yielded a large number of females, and therefore the F_1 females of only a limited number of P_1 could be used for detecting lethals. The largest numbers of F_1 vials from one P_1 vial for control, high and low anabolism groups were 24, 31, and 11, while the average numbers were 18, 11, and 6 respectively.

Results/

III. Results.

1. General survey of the results in both sexes considered together

The frequency of sex-linked lethals was determined both in the chromosomes derived from the males and in those derived from the females and, by the nature of the technique, equal numbers of both derivations were scored. Table I is a summary of the results in both sexes taken together, for each of the three groups of each of the three series, i.e. control, high anabolism and low anabolism. The first series was purposely kept smaller in the number of flies tested until it should become better known, through these preliminary results, what numbers it would be necessary to attempt to obtain in the three groups. Owing to the lowness of the rates of mutation found in this series, especially in the aged groups, it was decided to attempt to get considerably larger numbers and the second series was therefore made as large as possible. But as it did not prove feasible to get as many flies for either of the aged groups as in the controls in this series, and as it was not worth while from a statistical standpoint to have more flies in the one group than in the others, it was arranged/

arranged that in the third series, while the number of chromosomes examined in the aged groups was kept as high as in the second series, the number of chromosomes in the control was reduced nearly to half.

In the control, the total number of chromosomes examined, derived from both males and females, was 17,182. They contained 78 sex-linked lethals. The pereentage of spontaneous lethals - 0.45% - lies well within the range of the frequency of lethal mutations found in untreated material by Muller (1928), Demerec (1937) and many others, this range being approximately from 0.1% to 1%. The variation in the percentage of sex-linked lethals from one series to the other in the control is quite insignificant. The variation is also insignificant from series to series both in the high and in the low anabolism groups.

In the high anabolism group, the total number of chromosomes examined was 8,840, and these contained 15 sex-linked lethals, or only 0.17%. The difference between this result and that for the control is 0.28, which is more than 4 times its own standard error. Thus it appears that the frequency of sex-linked lethal mutations is lower in/

Table I.

Frequency of sex-linked lethal mutations in relation to age and anabolic activity.

(Combined results from males and females.)

No. of series	Control				High anabolism				Low anabolism				$P_a - P_b \pm \varepsilon d_1$ (d_1)	$\frac{d_1}{\varepsilon d_1}$	$P_a - P_c \pm \varepsilon d_2$ (d_2)	$\frac{d_2}{\varepsilon d_2}$	$P_b - P_c \pm \varepsilon d_3$ (d_3)	$\frac{d_3}{\varepsilon d_3}$
	No. of P_1	No. of chromosomes examined (each F_1 gave 2 chroms.)	No. of leth.	Percentage (P_a) $\pm \varepsilon$	No. of P_1	No. of chromosomes examined (each F_1 gave 2 chroms.)	No. of leth.	% $\pm \varepsilon$ (P_b)	No. of P_1	No. of chromosomes examined (each F_1 gave 2 chroms.)	No. of leth.	% $\pm \varepsilon$ (P_c)						
1	93	3466	18 (2 FROM SAME SOURCE)	.52±.12	92	1218	3	.25±.14	142	1418	3	.21±.12	.27±.18	1.5	.31±.18	1.7	.04±.18	.22
2	264	9210	41	.44±.07	152	4406	7	.16±.06	214	3280	5	.15±.07	.28±.09	3.1	.29±.1	2.9	.01±.09	.11
3	129	4506	19 (2 FROM SAME SOURCE)	.42±.09	141	3216	5	.16±.07	265	3240	6	.19±.08	.26±.11	2.3	.23±.12	1.9	.03±.11	.27
Total*		17182	78	.45±.05		8840	15	.17±.04		7938	14	.18±.05	.28±.06	4.6	.27±.07	3.9	.01±.06	.16

*On account of the agreement in the stocks and culture conditions in all three series corroborated by their agreement in mutation frequency, it was considered sufficiently accurate in this and the following tables to sum the data from all the series of each group together in the way appropriate for random samples drawn from the same lot of material, and to calculate the error of the totals on the same basis.

in old flies that have been kept in a condition of good nutrition and high reproductive activity than in young ones. The same seems to be true with the low anabolism group. Here there were 14 lethals in 7,938 chromosomes, or 0.18%, giving a difference from the control of 0.27, a value which again is 4 times as great as its own error. On the other hand, the difference between the percentages of mutations for high and low anabolism is only 0.01%, which is quite insignificant.

When the results of the different groups within the individual series are compared with each other, it may be noted that in the second series both the differences between the control and high anabolism frequencies and those between the control and low anabolism frequencies are significant. Although in the first series these differences in percentage appear almost as great as in the second series, they are not in themselves significant because of the small total numbers of chromosomes in the aged groups, while in the third series these differences verge on significance. In no series, however, is the difference between the high and low anabolism frequencies marked or significant.

It would be premature to attempt to draw/

draw conclusions from these results before examining separately the data for the chromosomes of maternal and paternal origin, respectively, for it is obvious that the conditions of germ cell production differ considerably in the two sexes in these experiments and that any changes in mutation frequency might fail to run parallel in them.

2. Effect of age and activity of cell multiplication in the female

Table II shows the results for the chromosomes coming from the females above. The control shows 32 lethals in 8,591 chromosomes, or 0.37%, the variation from one series to another being insignificant. The high anabolism shows 8 lethals in 4,420 chromosomes or only 0.18%. In this case the first series gives the mutation rate as high as 0.33% but as the number of chromosomes examined in this series is very small, the difference between the percentage of this series and that of the others is not significant. In the low anabolism 6 lethals were found among 3,969 female chromosomes, or 0.15%, again with insignificant variation from series to series.

The/

Table II.

Frequency of origination of sex-linked lethals in females (of wild type, F1 4 stock) in relation to age and anabolic activity.

No. of series	Control				High anabolism				Low anabolism									
	No. of P_1 vials	No. of chromosomes examined	No. of lethals	% of total (P_a) $\pm\varepsilon$	No. of P_1	No. of chromosomes examined	No. of lethals	% (P_b) $\pm\varepsilon$	No. of P_1	No. of chromosomes examined	No. of lethals	% (P_c) $\pm\varepsilon$	(P_a-P_b) $\pm\varepsilon d_1$ (d_1)	$\frac{d_1}{\varepsilon d_1}$	(P_a-P_c) $\pm\varepsilon d_2$ (d_2)	$\frac{d_2}{\varepsilon d_2}$	(P_b-P_c) $\pm\varepsilon d_3$ (d_3)	$\frac{d_3}{\varepsilon d_3}$
1	93	1733	5	.29	92	609	2	.33	142	709	1	.14						
2	264	4605	18	.39	152	2203	4	.18	214	1640	2	.12						
3	129	2253	9 (2 FROM SAME SOURCE)	.40	141	1608	2	1.2	265	1620	3	.19						
Total	486	8591	32	.37 $\pm$.06	385	4420	8	.18$\pm$.06	621	3969	6	.15$\pm$.06	.19$\pm$.08	2.4	.22$\pm$.08	2.8	.03$\pm$.08	.38

The difference between the percentages in the control and in the high anabolism groups, 0.19%, is 2.4 times its own standard error, while the difference between the control and the low anabolism, 0.22, is 2.8 times its standard error. It is therefore very probable that the control frequency is higher than that of either of the aged groups. On the other hand the difference between these groups is well below being significant.

These results then make it probable that the chromosomes transmitted by old females contain a lesser proportion of mutations than those transmitted by young ones. And despite the different conditions of food, anabolic activity, etc., under which the two groups of aged females were kept, there does not seem to be any considerable difference in their rate of mutation.

3. Effect of ageing sperm and of ageing the males by which sperms are produced

Table III gives the results for the chromosomes derived from the male. The control result of 46 lethals in 8,591 chromosomes, or 0.54%, is somewhat higher than that for the chromosomes/

Table III.

Frequency of origination of sex-linked lethals in male germ cells (of sc^8 sn w^a stock) in relation to age and anabolic activity.

	Control				High anabolism				Low anabolism									
No.of series	No.of P_1	No.of chrom-osomes ex-amined	No. of let-hals	% $\pm\varepsilon$ (P_a)	No. of P_1	No.of chrom-osomes ex-amined	No. of let-hals	% $\pm\varepsilon$ (P_b)	No. of P_1	No.of chrom-osomes ex-amined	No. of let-hals	% $\pm\varepsilon$ (P_c)	(P_a-P_b) $\pm\varepsilon d_1$ (d_1)	$\frac{d_1}{\varepsilon d_1}$	(P_a-P_c) $\pm\varepsilon d_2$ (d_2)	$\frac{d_2}{\varepsilon d_2}$	(P_b-P_c) $\pm\varepsilon d_3$ (d_3)	$\frac{d_3}{\varepsilon d_3}$
1	93	1733	13 (1 FROM SAME SOURCE)	.75	92	609	1	.16	142	709	2	.28						
2	264	4605	23	.50	152	2203	3	.14	214	1640	3	.18						
3	129	2253	10	.44	141	1608	3	.19	265	1620	3	.19						
Total	486	8591	46	.54±.08	385	4420	7	.16±.06	621	3969	8	.20±.07	.38± .10	3.8	.34±.11	3.1	.04±.09	.4

chromosomes from the females (0.37%). The high anabolism group showed 7 lethal mutations in 4,420 chromosomes, or 0.16%, while the low anabolism showed 8 in 3,969, or 0.20%. The difference between the control and high anabolism results, 0.38, is 3.8 times its standard error, while the difference between the control and low anabolism results, 0.34, is 3.1 times its standard error. Again the results of high and low anabolism are sensibly the same, the difference between these frequencies being quite insignificant.

It is evident that the trend of these results is very probably like that of the previous ones, in indicating that the frequency of lethal mutations in the control group is higher than in either of the aged groups.

4. Comparison of results in the two sexes

It is not legitimate to compare frequencies of mutations in male and female because of the difference in stocks used. One might, however, compare the effects of ageing on the mutation frequency in the two sexes, but the results are not extensive enough to allow any but a considerable difference in these effects to be noted. Nevertheless, there is a distinct suggestion in the results that the reduction in the mutation frequency with age (high anabolism group as compared with controls) is more pronounced in the males than in the females. In the low anabolism group such

Table IV.

Comparison of the reduction of mutation frequency in germ cells of males and females with ageing.

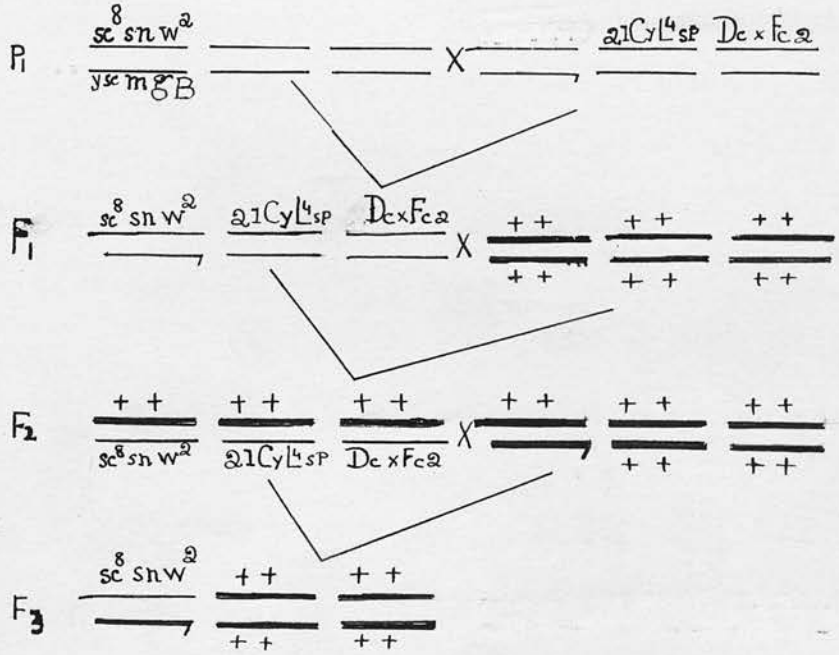
Sex of parent supplying chromosomes tested	$(P_a)\pm$ Control % of lethals	$(P_b)\pm$ High anabolism % of lethals	$(P_c)\pm$ Low anabolism % of lethals	$(P_a-P_b)\pm\varepsilon d_1$ (d_1)	$\frac{d_1}{\varepsilon d_1}$	$(P_a-P_c)\pm\varepsilon d_2$ (d_2)	$\frac{d_2}{\varepsilon d_2}$
female	.37±.06	.18±.06	.15±.06	.19±.08	2.4	.22±.08	2.7
male	.54±.08	.16±.06	.20±.07	.38±.1	3.8	.34±.1	3.4

$♂(P_a-P_b)\pm\varepsilon d$ (d_1)	$♀(P_a-P_b)\pm\varepsilon d$ (d_2)	$(d_1-d_2)\pm\varepsilon d_1$	$\frac{d_1-d_2}{\varepsilon d_1}$	$♂(P_a-P_c)\pm\varepsilon d$ (d_3)	$♀(P_a-P_c)\pm\varepsilon d$ (d_4)	$(d_3-d_4)\pm\varepsilon d_2$	$\frac{d_3-d_4}{\varepsilon d_2}$
.38±.1	.19±.08	.19±.13	1.5	.34±.11	.22±.08	.12±.13	.9

a difference between the sexes is much less marked, and quite without significance.

5. Some results of using sc^8 B w^a instead of sc^8 sn w^a males

After the experiment had been started, it was considered more advantageous for the sc^8 sn w^a stock to have its 2nd and 3rd chromosomes derived from the F1 4 stock and in homozygous condition. The scheme used for the preparation of such stock was the following:



Such males were maintained by crossing them with ♀ ♀ females which were made at the same time in such a way as to have their 2nd and 3rd chromosomes from the F1 4 stock and in the homozygous condition.

These crosses were made at the same time as the three series, utilizing the ordinary sc^8 sn w^a, previously reported, were being obtained. After these preparatory crosses had been carried through, these flies were used for a time in the place of the ordinary sc^8 sn w^a stock until it was realised that unfortunately the sc^8, including its inversion, had been lost from this stock by a rare type of double crossing over. The results obtained with the latter flies were rejected. In the new series started after that sc^8 B w^a males were used in the P_1 generation.

Two series have so far been carried through with the use of sc^8 B w^a P_1 males. The results of these series (shown in Table V) go to support the results previously obtained. It will be seen that in the control the total number of tested chromosomes of maternal origin was 3,516, which included 11 lethal mutations. The percentage, 0.31, is sensibly the same as found in the previous experiments. In the high anabolism the corresponding/

Table V.

Summary of results with sc^8 B w^a males crossed to Fl 4 females.

sex of parent supplying chromosome tested	Control			High anabolism			Low anabolism		
	No. of chromo-somes	lethals	%±εd	No. of chromo-somes	lethals	%±εd	No. of chromo-somes	lethals	%±εd
female	3516	11	.31±.09	1892	4	.21±.1	1673	2	.12±.08
male	3516	4	.11±.05	1892	1	.05±.05	1673	2	.12±.08

corresponding numbers are 4 lethals in 1,892 maternally derived chromosomes, or 0.21%. Although the difference between the mutation rate here and that in the control is not significant, because of the small numbers, the result agrees with the earlier ones as well as was to be expected. Similarly in the low anabolism series the results for the maternally derived chromosomes - 2 lethals in 1,673 chromosomes, or 0.12% - also agrees well with the earlier results.

Examination of the behaviour of the male chromosomes in the three groups shows a much lower rate of mutation in this stock than in the one previously used. Unfortunately, because of this, the total numbers of paternally derived lethals are too small to allow any significant comparison to be made between the different groups. The negative statement may however be made that there is again no evidence of any higher mutation frequency either in the aged sperm or in sperm derived from old males, than in fresh sperm from young males.

6. Appearance of a visible yellow mutation in the sc^8 B w^a chromosome

In the low anabolism group, a yellow mutation/

mutation arose in the sc^8 B w^a chromosome. This yellow, designated as y^{Si}, is phenotypically like the typical (extreme) yellow and the stock thus provided is so far as we know the only sc^8 stock available containing a typical yellow, the others with yellow alleles (when not accompanied by a simultaneous change in the achaete character, indicating a minute deletion or other structural change) having dark bristles. In this stock (y^{Si} sc^8 B w^a) the crossing-over seems to be of the usual frequency for the sc^8 chromosome.

Discussion/

IV. Discussion.

From the results given above it seems clear that, temperature being constant, the ageing of female and male with high anabolic processes (allowing enough yeasted food and mating), ageing of female with low anabolic processes (on syrup food) and ageing of sperms inside the body of the female with low anabolic processes, decreases the frequency of sex-linked lethal mutations.

Experiments of a similar type, but with different results, have been reported by Sacharov (1939), by Timofeeff-Ressovsky (1937) and by Shapiro (1938). Sacharov found a significant increase in the rate of mutation in aged sperm (30 days with temperature 18°C) as compared with that in the non-aged sperm, and regards his results as giving evidence of an accumulation of mutations during the ageing of the sperm. He also conducted experiments with paternally derived chromosomes from males and inseminated females in a state of "hibernation" (at 5° to 7°C temperature), and these results too indicated a rise in the rate of mutation attendant upon the ageing of the sperm and of the males (the tables do not differentiate between the two groups).

The/

The difference between Sacharov's "hibernation" experiment and ours may be due to the difference in temperature. It has been reported by Kerkis (1939) and by Birkina (1938) that very low temperature increases the rate of mutation in sperm and this factor may well have caused the increased frequency of mutation observed by Sacharov in his low temperature experiment, although it would not be expected to apply to the experiment at 18°C. However, it is difficult to comment thoroughly upon these results which have been given without the full details of the actual technique and food applied.

Timofeeff-Ressovsky (1937) also has reported that the ageing of sperms (at 22°C) increases the rate of mutation. The chief difference between his experiment and ours on sperm seems to be the duration of the ageing which was 20 days in his experiment and 26 days in ours. Here again we must reserve judgment as to the cause of the discrepancy. Shapiro's experiment (1938), in which he used 25°C temperature for the control flies and 14°C for the experimental ones, showed, to be sure, some increase in the rate of mutation of the aged sperms, but the difference does/

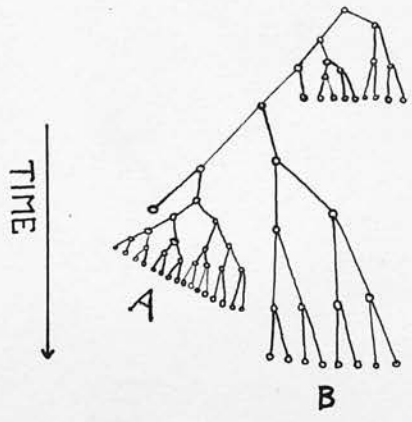
does not look statistically significant. If his data are carefully examined and correctly calculated, it may be found not only that the mutation rate does not increase according to the age of the spermatozoa but also that there does not seem to be any marked effect of age on the rate of mutation.

In order to judge of the bearing which our results may have on the general problem of the mechanism of mutation and its relation with age, it is desirable to consider in what parts of the life cycle the mutations occur. Muller's results (1920) showed that mutations can occur at any time and place during the life cycle. Our present results may throw some light on the problem of the relative frequency of mutations at different stages of the germ tract. It is important to see how the cells in the germ tract divide. Do they have the same frequency of division at all times? Is this frequency influenced by the position of the cells in the germ tract or not? These questions require solution in connection with the problem of how the frequency of mutation may vary in different stages of the life cycle of the animal. Muller (op. cit.) constructed a hypothetical diagram of the cell lineage of the germ tract, showing the/

the length of time that each cell remained "at rest" in every cell generation, as well as the number and distribution of the gametes which subsequently underwent fertilization and development. The pattern of cell lineage shown in such a diagram, and the time relations and mutation frequencies existing in its different parts would all represent important factors in the determination of any differences in the rate of mutation found in testing gametes obtained at different developmental stages.

The diagram in Fig. 1 may be assumed to represent roughly and in simplified fashion the cell lineage of a parent individual (P_1 male or female). This diagram starts with the "primordial germ cell" of the parent and may be taken to end in the formation of the gametes which were used for the control experiments and also for those in the aged groups (high and low anabolism). The separation between two groups of cells has purposely been exaggerated: one of these (A) represents the derivation of the cells which gave the control F_1 flies and the other (B) represents the derivation of those which produced the F1 flies of the aged groups. The necessity of postulating/

FIG. 1



HYPOTHETICAL DIAGRAM OF THE CELL LINEAGE OF THE GERMTRACT

postulating differences of the kind shown arose from the consideration that if the F_1 flies of the aged series had been produced by a mere continuation of the process of division of the same group of germ cells from which the control F_1 flies had come, then the rate of mutation for the aged group should either be equal or, most probably, should be higher than that in the control. The results, showing that this is not the case, may be interpreted best by us assuming that the cells of these two groups were highly independent of each other, that the "A" group cells were more active in the early part of the life of the P_1 flies, when they produced gametes for the control flies, and that the "B" group cells, although they must have originated at the same time as those of group "A", were more or less dormant at first and became active only after the flies had been aged for a considerable period. This comparative dormancy which is assumed to have delayed the cell division and mutation of these germ cells might at the same time have caused the low rate of mutation found in the cells from the aged flies.

If the above assumption regarding the germ cell lineage is right, it could lead us to infer/

infer that the cells in the germ tract may differ from place to place as well as from age to age with regard to their "spontaneous" mutability.

The above interpretation applies to the results from the aged females and males, but it is not immediately evident what bearing it could have on those from the sperm aged in the spermathecae of the females. This question then requires separate consideration. In this connection we may first consider the results of some previous work, dealing with mutations induced in spermatozoa by X-rays.

In a study of the influence of ageing of sperm (in the male) after irradiation on the frequency of X-ray induced mutations found in them, Harris (1929) found no perceptible change in mutation frequency in the aged sperm as compared with freshly irradiated sperm. Lobashov (1938), however, reported results which showed that the rate of induced mutations diminishes in the sperm aged (in the female) after irradiation. He concludes that a selective elimination of mutated spermatozoa occurs in this case. Such a conclusion is at variance with the results of Muller and Settles (1927), who showed that it is highly/

highly unlikely that either natural or artificial agents can cause a selective elimination of spermatozoa on the basis of their gene content.

Our present results show a decrease in the frequency of "spontaneous" mutations associated with the ageing of spermatozoa in the spermathecae. During this process of ageing, the majority of surviving spermatozoa must have been lost or had the opportunity of fertilizing eggs and only a few remained stored till the 25th day after insemination. It would be of interest here to find out whether there was not a particular kind of spermatozoa which did not get the chance of fertilizing eggs and which hence underwent the ageing. Was their delay in functioning due entirely to their position in the spermathecae or was it in part at least due to their own peculiar physiological constitution? The solution of these questions would throw much light not only upon our own results but also upon those obtained by Timofeeff-Ressovsky, by Sacharov, by Shapiro, and by those workers who studied the influence of ageing of spermatozoa on the frequency of mutations induced by radiation. If the long stored spermatozoa did not differ from the/

the fresh ones in their mutability, the rate of spontaneous mutation found in the aged sperms should have been even higher than that found in the fresh ones, since in addition to having the mutations of the young sperm they would have had those which had accumulated in the meantime. Our results, then, show that, either because of the position which the spermatozoa have in the spermathecae or because of their own physiological constitution which would also make them less active in reaching and fertilizing the eggs, the sperms which remain to fertilize the eggs of the aged fly have an unusually low rate of sex-linked lethal mutations.

It is of interest to compare the duration of the reproductive generation of the flies (roughly 2 weeks) with that of human beings, which may be taken as thirty years. The ratio of thirty years to two weeks is about 750:1, of the order of one thousand. In human beings the spontaneous mutation rate of very few genes is known (Haldane, 1935), but in those known it is of the same order as for the Drosophila genes studied. This would lead to the conclusion that the mutability per unit of time/

time in slow breeding animals is much lower than in Drosophila. Had it not been so all the chromosomes of the slow breeding animals would be full of lethals within a few generations (Muller and Altenburg, 1919). Perhaps the cause of this phenomenon is to be found in the same factors as those which cause the mutation frequency of aged Drosophila to be no higher, or even lower, than those of young individuals.

Conclusion/

V. Conclusion.

It may be concluded from results and considerations above set forth that the frequency of sex-linked lethal mutations in Drosophila is reduced significantly in old age. These results, if applicable to domestic animals, would be of considerable significance in animal breeding, since it might in the long run be advantageous to reduce the mutation frequency here, in view of the fact that most mutations, whether occurring "spontaneously" in nature or induced by X-rays, temperature or otherwise, are deleterious (Muller, 1918, 1921, 1927; Patterson and Muller, 1930).

Summary/

VI. Summary.

A study was made of the effect of age and anabolism on the frequency of sex-linked lethal mutations in Drosophila melanogaster. Two sets of experimental flies were used; the first eggs laid by them furnished the control material. In one experimental set the males and females were kept together individually on standard yeasted food (high anabolism), and in the other set the females, after being inseminated, were removed from the males and kept on syrup food (low anabolism). The flies of both sets were transferred every 4th day till the 26th and 25th days, respectively, after mating, when they were finally placed on standard yeasted food for the laying of eggs. The flies (F_1) derived from these eggs were tested for the presence of sex-linked lethals. Temperature (23°C) and other conditions were maintained as constant as possible.

The following are the results:-

1. The frequency of sex-linked lethals is reduced significantly in both the male and female germ cells produced in old age, as compared with those produced while young.
2. Old age combined with low anabolism of the germ/

germ cells seems to result in about the same degree of reduction in the frequency of lethal mutations in female germ cells as old age combined with high anabolism (adequate food and mating).

3. The ageing of the sperm inside the spermathecae of females having low anabolic processes gives a lower rate of sex-linked lethals than that found in the non-aged sperm.
4. There is some indication of a tendency for the frequency of lethals to be reduced more by ageing in the case of the male than of the female germ cells.

Acknowledgments/

Acknowledgments.

The author wishes to express his indebtedness and appreciation to Professor F.A.E.Crew and Dr A.W.Greenwood for their hospitality, and to Dr H.J.Muller for his advice and guidance throughout the course of this study.

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SACHAROV/

IX. All candidates for the degree of D.Litt. shall present a thesis or a published memoir or work,[1] which shall be an original contribution to learning, to be approved by the Senatus on the recommendation of a committee appointed by the Senatus; provided that, if required by the Senatus, a candidate shall also be bound to pass such examination, conducted orally or otherwise, on the subject of his thesis or of his special study as may from time to time be determined. The thesis, or memoir, or work shall be accompanied by a declaration signed by the candidate that it has been composed by himself. If the thesis has not already been published, it shall be published by the candidate in such manner as the Senatus shall approve.

General.

X. Notwithstanding and in supplement of the Regulations as to Examinations, Sections XIV. and XV., the Senatus Academicus in each university shall appoint such Professors or Lecturers as it may think suitable to conduct the examination of candidates who may offer themselves under the Regulations for Degrees in Science, or of these Regulations for the Degree of Doctor of Science, or Doctor of Letters; and the University Court shall, after consultation with the Senatus Academicus, appoint an additional examiner to act along with them. Such additional examiner shall be a person of recognised eminence in the subject of the thesis, or memoir, or work which is to be submitted for approval. The result of the examination shall be reported to the appropriate Faculty or committee of Senatus.

XI. The degrees of D.Sc. in Mental Science or D.Sc. in Philology shall no longer be conferred by any Scottish University.

XII. The degrees of D.Sc. and D.Litt. shall in no case be conferred on persons who have not satisfied the conditions hereinbefore set forth, and shall not be conferred *honoris causa tantum.*

The fee to be paid for examination for each of the degrees of D.Sc. and D.Litt. is £15, 15s., payable when the thesis is lodged, and the fee for each re-examination for each of the degrees is £5, 5s.

Candidates for examination or graduation must pay the matriculation fee of £2, 12s. 6d. Candidates for graduation are required to enter their names and (if not previously registered) to pay the registration fee at least three days before the date of the Graduation Ceremonial.

DOCTOR OF PHILOSOPHY (Ph.D.)[2]

I. A degree of Doctor of Philosophy (Ph.D.) may be conferred by the University of Edinburgh in any Faculty.

II. The degree shall be open to graduates of this University or of any other approved university, or, in exceptional cases, to persons possessing qualifications approved by the Senatus for this purpose.

III. A candidate for the degree shall be not less than 21 years of age at the time of his admission to study for the degree. He shall previously have pursued a course of study extending over not less than three years at a university or institution of university rank.

IV. It shall be the duty of the Senatus Academicus:—(1) To deal with all applications for admission to prosecute special study or research with a view to the

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[1] Two copies of the *text* of the thesis (plans and illustrations need not be duplicated) must be lodged and the fees paid by 1st January for the summer graduation, and by 1st June for the December graduation.

[2] University Court Ordinance No. 28.

degree; (2) to make regulations for the supervision of the work of students under the Ordinance; (3) to satisfy themselves from time to time that the students under the Ordinance are carrying on their work in the University in a satisfactory manner; (4) to suspend or exclude from any course any student whose conduct or progress is unsatisfactory.

V. Every applicant for admission must make to the Senatus Academicus a written application,[1] stating the degree or other qualification which he possesses, the line of special study or research which he wishes to prosecute, and the probable period of its duration, and submitting evidence as to capacity and general qualifications.

VI. The Senatus shall not admit any applicant unless they are satisfied:—(*a*) That his previous training has been suitable; (*b*) that he is qualified to prosecute the proposed line of special study or research; (*c*) that the proposed line of special study or research is a fit and proper one; (*d*) that he proposes to prosecute his special study or research during a period to be approved, which shall be not less than two academical years.[2]

VII. Every such student shall be required to matriculate each year, paying the ordinary fee.

VIII. The University Court may provide such sums as it may think fit in aid of the expenses of special study or research under this Ordinance.

IX. At the close of the period of special study or research, the candidate shall present a thesis embodying the result of his work, which shall be submitted to examiners nominated by the appropriate Faculty or any committee entrusted by the Senatus with the supervision of the student's work, and one of such examiners shall be an external examiner appointed by the University Court. The examiners shall, in addition to reading the thesis, conduct such written or oral examination as the Faculty or committee may from time to time prescribe.

X. The report of the examiners shall be made, in the first instance, to the appropriate Faculty or committee, as the case may be, who shall report to the Senatus whether the candidate is, or is not, worthy of the degree.

XI. The fee payable on submission of the thesis for the degree shall be £10, 10s.

XII. The degree shall in no case be conferred on persons who have not complied with the conditions hereinbefore set forth, and shall not be conferred *honoris causa tantum.*

Additional Regulations.[3]

1. Applications for admission as Ph.D. students must be sent on the official form to the Secretary of the University during the month preceding one of the following dates:—1st October, 1st January, and 1st April in each year. Applications for admission will be considered in the first instance by the heads of the departments in which the proposed subjects of research lie (in Theology, by the Board of Studies in Theology), who will make recommendations to the Ph.D. Committee of the Senatus regarding admission and supervision of candidates.

2. The subject of research must be one for which expert supervision can be provided within the University of Edinburgh.

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[1] Applications will be considered on one occasion in each term. They must be lodged at or before the following dates:—1st October, 1st January, and 1st April.

[2] See Additional Regulations, par. 5, *infra.*

[3] Made by the Senatus under Section IV. (1) and (2) of the above Ordinance.

3. The Ph.D. Committee will prescribe for each candidate a period of study, which will usually be either six terms or nine terms. Six terms are required as a minimum for those who are giving the whole of their time to their research. In the case of candidates who are members of the University staff, the Committee may determine whether the period shall be nine or six terms. Others who can only devote part of their time to research will be required to take nine terms. Candidates working for the degree of Ph.D. are in no case allowed to take courses with a view to any other degree or diploma during their period of study for the doctorate.

4. Candidates may take longer over the preparation of their theses than the minimum period assigned, but every thesis must be presented within five years from admission as a candidate. The ordinary matriculation fee of £2, 12s. 6d. must be paid for each year from admission to graduation.

5. All candidates must prosecute their research under a supervisor in the University of Edinburgh, and, except as provided in rule (*b*) below, must be in residence during the whole of their period of research. Special leave to work elsewhere may be granted for a portion of the prescribed time, when some of the material necessary for the research is not available in Edinburgh. The granting of this leave will be governed by the following rules :—

(*a*) In the case of graduates of the University of Edinburgh for whom six terms have been prescribed, the absence must not exceed three terms if the degree is to be obtained at the end of the six terms. Should the work call for a longer absence, the period of research must be correspondingly increased, but if the consequent total time should reach nine terms, the candidate will fall under rule (*b*) below.

(*b*) No definite conditions of residence are imposed on those graduates of the University of Edinburgh who are taking nine terms, but the Ph.D. Committee will satisfy itself that supervision from Edinburgh is exercised.

(*c*) In the case of candidates who are not graduates of the University of Edinburgh, for whom six terms have been prescribed, the absence shall not exceed two terms out of the six, and these must not include either the first or the sixth term of the period of research. Should the work call for a longer absence, the period of research must be correspondingly lengthened.

6. Candidates who are not devoting their whole time to their research must report in person to their supervisors at least twice in each term, unless leave of absence has been granted.

7. The Ph.D. Committee shall receive annual reports from supervisors on the work of all candidates.

8. Candidates desiring to graduate in the summer term must give notice on the official form to the Secretary of the University and pay the fee of £10, 10s. by 10th April, and must present the thesis before 15th May. For the December graduation the corresponding dates are 1st October for notification and 1st November for the presentation of the thesis. Two copies of the thesis are to be sent to the Secretary.[1]

9. In the natural sciences the thesis is expected to give such new facts, with experimental or other evidence, as would form material for the publication of a paper in a scientific journal. In the humanistic studies the thesis should be a distinctive work of learning or research, of independent critical judgment, worthy of publication as a thesis approved by the University.

10. A typed copy of every approved thesis will be deposited in the University Library.[2]

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[1] When the thesis includes expensive diagrams, etc., a single copy of such portions of the thesis will be accepted ; but, where possible, photographic copies of the originals should accompany the second copy of the thesis.

[2] It is requested that theses be presented in large quarto form.

11. Should the thesis be substantially satisfactory, as attaining a sufficiently high standard in both matter and form, and yet, in the opinion of the examiners, call for modification in certain respects, the making of certain defined changes may be required as a condition antecedent to graduation. In such cases the candidate will be admitted to graduation only upon receipt of a statement, signed by the internal examiner or examiners, that this requirement has been fulfilled.

12. No thesis regarded as unworthy of the degree can be resubmitted unless the Ph.D. Committee invites the candidate to present it in a revised form. A fee of £5, 5s. shall be paid for such resubmission.

13. When the Ph.D. Committee deems it expedient, an examination of a candidate may be held, after the presentation of the thesis.

14. Candidates are normally expected to possess, or to acquire, a reading knowledge of French and German, but the Ph.D. Committee may impose such alternative linguistic requirements as the nature of the study demands. Failing satisfactory documentary evidence of such reading knowledge, candidates will be tested by an examination in languages, which may be taken on admission as a Ph.D. student, or in the course of the first year of study, but in no case later than the beginning of the second year of study. It will be conducted jointly by the supervisor and a member of the appropriate linguistic department. In the event of failure to pass this examination at the beginning of the second year, the period from that failure up to the successful passing will not be counted as a part of the allotted time devoted to the research.

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II.

INFLUENCE OF PROLONGATION OF THE LARVAL LIFE ON THE OCCURRENCE OF SPONTANEOUS MUTATIONS IN DROSOPHILA

I. Introduction.

Our previous results which showed that the frequency of spontaneous sex-linked lethals is reduced significantly in the germ cells produced in old age as compared with those produced in young flies were interpreted by assuming that peculiarities differentiating different parts of the germ tract and their rate of germ cell division might be playing some part in influencing the rate of spontaneous mutation. The germ cells which were examined in our previous experiments were all subject to the same normal conditions during the pre-imaginal stages, but differed in respect of their time of production by the adult, or their time of functioning. The question next arose whether the prolongation of a different stage of the fly's life could have an influence on the rate of spontaneous mutation.

To decide such a question as well as to see whether further light could be thrown on our hypothesis concerning the relation of the nature of germ tract to the mutability of the germ cells, an experiment was undertaken to test the effect of prolongation of the larval life, brought about by retardation of growth, on the frequency of sex-linked/

linked lethal mutations.

II. Methods and Material.

Prolongation of larval life.

At a temperature of 23°C the fly emerges from its pupal case some ten days after the egg is laid, when standard yeast food (Offermann and Schmidt, 1935) is used in 4 by 1 inch vials containing one pair of parents. For the purposes of the present investigation, this period of ten days was to be prolonged as much as possible or at least to about 4 weeks.

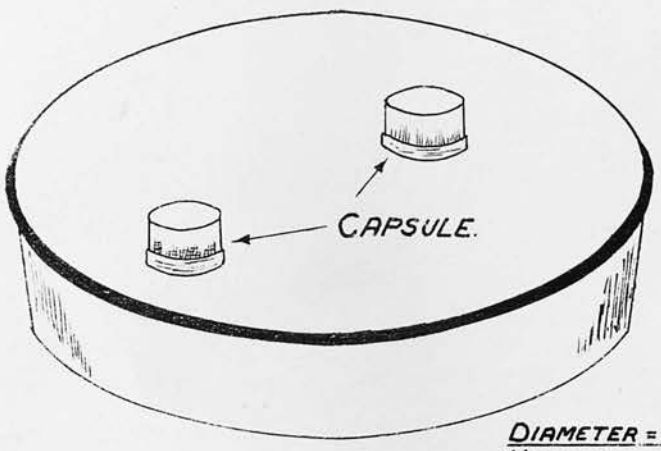
A number of studies have been published concerning the relation of longevity in flies to particular experimental conditions, such as temperature variations and other environmental factors, increased muscular activity, different kinds of food and continuous starvation with or without water (e.g. Pearl and Parker, 1921-24, Kopec, 1928, and Sekla, 1928). A few of them report the prolongation of the whole period of development by 24 to 30 per cent. by feeding larvae only every second day or on meagre food.

After/

After our work had been begun, Beadle, Tatum and Clancy (1938) published some results regarding the effect of food on the development of larvae. These results show that larvae deprived of food at any time up to the stage normally reached 70 hours after egg laying are retarded in development, and starvation at a later stage affects the size and eye-colour of the fly.

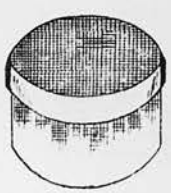
Syrup food (Offermann and Schmidt, 1935), which is considered best for preserving the adult flies for a considerable time, presented itself as probably the most promising medium for prolonging the larval period also. A few preliminary experiments had to be carried out, however, to determine the conditions best suited for the laying of the eggs, and for periodic transfers of the larvae, before the method finally standardized, as described below, was devised.

Fresh males and females were mated together individually in vials containing standard food. After 72 hours the females were separated from the males and placed in small capsules (Fig. 1) which had netting on one side and were closed on the other. The capsules were placed on standard food, not sprayed with live yeast, with their netting side down on the food, so that the flies might/



DIAMETER = 2·0 cm.
HEIGHT = 2·3 cm.

DISH WITH FOOD.



DIAMETER = 2·1 cm.
HEIGHT = 1·7 cm.

CAPSULE WITH FLY.

might take food and lay eggs through the netting. After the flies had laid a considerable number of eggs (18 hours were allowed) they were removed with the capsules and the eggs were transferred with the food to a vial. Twelve hours after the first hatching of the eggs, the larvae were picked off with needles under the binocular microscope and transferred to syrup food. On the 6th and again on the 12th day after this they were transferred to new syrup food. On the 17th day, they were transferred back to standard yeasted food for 18 hours. Then they were put on syrup food once more, where they pupated and emerged from the pupa case 27 to 30 days after the laying of the eggs.

It was noted that this system of under-feeding not only results in delay in the maturing of the flies (due, according to Beadle et al., to an effect on the first part of their developmental period) but also exerts considerable influence on their growth (due to an effect on the second part of larval development). The starved flies are far smaller in size and weight than the normal ones. Their eye colour is also lighter, when they are freshly emerged, than that of freshly hatched flies that had a normal developmental period/

period. 47 flies of the starved group and the same number of the control were weighed one day after emergence and their weights were 0.021 gms. and 0.057 gms. respectively.

Description of a typical experiment.

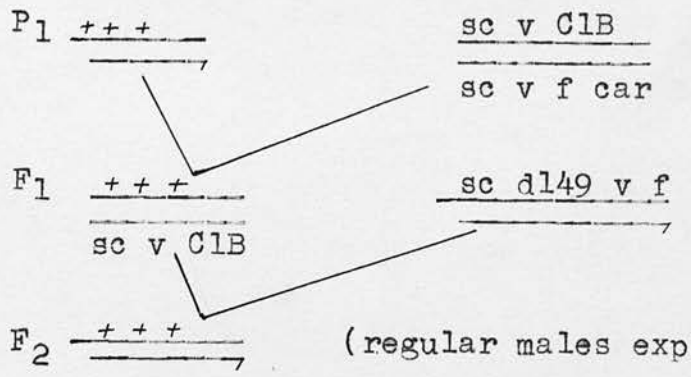
25 males and 25 females of "Florida 4" wild type homozygous stock, the same stock as used in our experiment on ageing of the adult, were mated individually in vials which were called "P_0", and were designated by numbers. They were kept in the P_0 vials for 72 hours, during which the females laid eggs, The flies which developed from these eggs (emerging some 10 days later) were designated as P_1, the males being used for the control group. Counts of the P_1 males and females in these vials were made to make sure that there was no lethal already present, derived from the previous generation.

The P_0 females, after having been inseminated and allowed to lay eggs for 72 hours in the above-mentioned vials, were removed from the males and placed immediately in capsules (having the same number as the P_0 vial from which the female was taken). These capsules were placed with their/

their netting down against standard food (without live yeast) which was in a dish (Fig. 1). The dish was covered by a tray so that no foreign flies might lay eggs on the food. 18 hours after the flies had been placed on this food, they were removed from it and the food, with its eggs, that had been under each capsule was cut out and placed in vials marked with the same number as had been given the capsule and the corresponding P_0 vial. 18 hours after such food with eggs was placed in the vials, the larvae were picked off and placed on syrup food which was put in vials marked with the same number. The later transferences, which were accomplished with the aid of two needles, followed the schedule already described. The males derived from these larvae were collected and were designated as "starved P_1". Each of them was given both the "P_0" number of its parents and a distinctive "P_1" number.

The lethal mutations arising in the control P_1 males as well as those in the starved P_1 males were detected by the ClB method. This involved/

involved the following crosses:



The absence of such males showed the presence of a lethal. Rarely one or two non-disjunctional males appeared in the vials to be scored as lethals. They were very easily recognized, however, as they had the markers sc v f or sc v f car. All the lethal determinations were verified by breeding the F_2 female of type $\frac{+ + +}{\text{sc d149 v f}}$ and noting whether wild type males were absent in their progeny (F_3).

When more than one case of a lethal was found among the F_1 derived from the same original P_1 male, these lethals were located by crossing over tests. If they were found to have the same locus they were considered to have been derived from the same original mutation.

It should be noted that during the entire period of breeding and raising of the P_0, P_1/

P_1 and F_1 of both the control and underfed groups the temperature was kept at 23°± 1°C.

III. Results and Discussion.

In all, 4 series were carried on. In the first 3 series the chromosomes derived from the P_1 males were tested for sex-linked lethal mutations by the ClB method while in the fourth series both chromosomes from the P_1 males and from their sisters (which here became "P_1 females") were tested. The tests of the females were conducted by crossing them to $\underline{sc^8\ B\ w^a}$ males and testing the F_1 females individually for the production of wild type sons (F_2).

In the control the total number of chromosomes examined (Table I) was 5,118 and lethals obtained 24, or 0.47%. In the starvation group 15 lethals were found among 4,448 chromosomes or 0.34%. The difference between the two percentages (0.13), being only 1.00 times its standard error, cannot be called significant. It may be noted here that in the control group, in two cases two lethal mutations were derived from the/

Table I.

Frequency of sex-linked lethal mutations in control flies and flies having their larval period prolonged by underfeeding.

sex of parent supplying tested chromosome	No. of series	Control (P_1 males emerged 10 days after eggs were laid) Temp. 23°C				Underfed group (P_1 males emerged 27-30 days after eggs were laid) Temp. 23°C								
		No. of chromosomes tested	No. of lethals	% of lethals (p_1)	± ε d (corrected for the cases appearing from the same source)	No. of chromosomes	No. of lethals	% of lethals (p_2)	± ε d (corrected for the cases appearing from the same source	$p_1 - p_2$ ± ε d (d_1)	$\frac{d_1}{\varepsilon d}$	expected on basis of p_1 result if frequency were proportional to time (p_3)	$p_3 - p_2$ (d_2)	$\frac{d_2}{\varepsilon d}$
male	1	847	6			740	6 (2 FROM SAME SOURCE)							
male	2	1052	5 (2 FROM SAME SOURCE)			907	1							
male	3	1112	6 (2 FROM SAME SOURCE)			1140	3							
male	4	1130	4			942	3							
female	4	977	3			719	2							
TOTAL		5118	24	.47	± .1	4448	15	.34	± .09	.13 ± .13	1.00	1.20 ± .32	.87	2.7

the same source, whereas in the starvation group 5 lethal mutations were all derived from one source. An approximate correction has been made for this circumstance in the calculation of the standard error, using the relation $\varepsilon_p : p :: \varepsilon_m : m$, where p is the frequency of mutations found and m is the number of separate occurrences of mutation divided by the number of chromosomes tested. This relation departs more and more from the actual the more uneven the distribution of "multiple" cases. In our case, for instance, the difference in the rate of mutation between the two groups would have been much more marked if only the one mutation of the primordial cell that produced 5 lethal-bearing F_1 had happened not to occur, during some very early stage of the germ cell proliferation in the underfed group.

If the occurrence of mutation were proportional to time, the expected percentage for the underfed group would be 1.20 ± 0.3. The difference between the expected percentage and that actually found in the underfed group is 0.87, which is 2.7 times its standard error. This difference is quite significant.

It is to be noted that these results agree in general with/

with those of Olenov (1939), which appeared after our own work had been in progress for some time. Olenov investigated lethal mutations in the X-chromosomes as well as in the second chromosomes in both the control and experimental groups. His results show no significant difference between the rates of sex-linked lethal mutations of the experimental flies (whose larval life was prolonged) and those of the control but they do indicate a somewhat higher rate of lethals in the second chromosomes derived from the experimental flies than in those from the control. The increase, however, is not nearly large enough to appear to be proportional to time. Thus Olenov's results and ours are seen to be in agreement regarding the most essential point investigated.

The most important point of our present work, as of that reported in our parallel paper, is that the rate of mutation does not increase in proportion to the prolongation of life no matter what stage has been extended; rather it shows a tendency to decrease if the time factor is taken into account. It appears that, during the process of prolongation of larval life by insufficient feeding, the germ cells were not able to multiply in/

in the same normal way as in the control flies, and that this inhibition of germ cell multiplication (involving lowered anabolic activity including lesser gene reduplication) is probably associated in some way or other with the lowered mutability of the germ cells.

It may be concluded that inhibition of growth resulting in prolongation of larval life and partial inhibition of germ cell multiplication indirectly reduces the likelihood of mutations occurring in these germ cells within a given time.

IV. Summary.

An experiment was undertaken to determine the effect of prolongation of larval life, that is, of retardation of growth, on the frequency of spontaneous sex-linked lethal mutations in Drosophila melanogaster. The control P_1 flies were bred on standard yeasted food and emerged some 10 days after the eggs had been laid, whereas the underfed flies were with the exception of two short intervals fed on syrup food from the first day of larval life till they emerged from the pupae 27-30 days after the eggs had been laid. The tempera-

ture/

ture (23°C) and the stock used (wild type Florida 4) were the same for both groups. Sex-linked lethal mutations were detected in the paternally derived chromosomes by the ClB method in four series and in the maternally derived chromosomes by a modification of this method in one series.

The results do not show a significant difference between the frequency of mutation in the control and in the underfed group, although there is an indication of a lesser frequency in the underfed group. When the duration of the generations in the two groups is taken into account, it is found that the underfed group has a significantly lower mutation rate, per unit of time, than the control group.

These results are in a general way confirmatory of our previous ones, in showing that mutation frequency does not rise proportionally with increase in age of the fly.

Acknowledgments.

The author wishes to express his indebtedness and appreciation to Professor F.A.E.Crew and Dr A.W.Greenwood for their hospitality, and to Dr H.J.Muller for his advice and guidance throughout the course of this study.

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